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14. ABSTRACT

Our research described here is relevant to the pathogenesis and a potential novel therapy for breast cancers. The PIK3CA is the most commonly mutated oncogene in breast cancer and loss of the tumor suppressor, PTEN, occurs frequently in patients suffering from this disease. The most significant accomplishment during the second year of funding is the mammary gland tumor model induced by conditional expression of an oncogenic PIK3CA allele which will allow us to determine the oncogenic role of PIK3CA in tumor initiation, progression, maintenance and metastasis etc. It should also significantly facilitate preclinical testing for the development of PI3K inhibitors for targeted therapy. Our final goal of simultaneous inactivation of PTEN and activation of PIK3CA will not only provide a new perspective on the relationship of the two key oncogene and tumor suppressor, PIK3CA and PTEN, and the signaling pathway under their control in cell regulation and oncogenic transformation, but also a potential novel therapy to all patients plagued with the common tumorigenic mutations.

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Annual Summary Report for Award Number W81XWH-06-1-0341

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Introduction

The lipid phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is a critical second messenger in cell signal transduction. The level of PIP_3 is tightly regulated by the activities of two opposing enzymes, phosphatidylinositol 3-kinase (PI3K) and Phosphatase and tensin homolog (PTEN), acting as "on/off" switches. We hypothesis that PI3K activity is tolerated within a relatively narrow window in cells - "too much of PIP_3 is just as lethal as too little". The abnormal elevation of PIP_3 levels has been frequently found in human cancers bearing somatic activating mutations in the *PIK3CA* gene or loss of PTEN function (Bachman et al., 2004; Samuels et al., 2004). Interestingly, while both *PIK3CA* and *PTEN* mutations occur so frequently in cancers, *PIK3CA* mutations and *PTEN* loss are almost mutually exclusive (Broderick et al., 2004; Byun et al., 2003; Saal et al., 2005). Since the two genes act as "on/off" switches on PI3K signaling, the reciprocal nature of *PIK3CA* mutations and *PTEN* inactivation indicate that while either *PIK3CA* activation or *PTEN* loss of function results in an elevation of PIP_3 sufficient for oncogenisis, the *PIK3CA*/PTEN double mutants may elevate PIP_3 to a lethal level. To test this hypothesis, we want to inactivate *PTEN* in cells expressing activating mutants of *PIK3CA*. We will also test our idea by simultaneous activation of PI3K and inactivation of *PTEN* in an animal breast tumor model.

Aim 1. To determine the effect of *PTEN* inactivation in human mammary epithelial cells (HMECs) expressing activated alleles of *PIK3CA.*

Aim 2. To determine the effect of simultaneous inactivation of *PTEN* and activation of *PIK3CA* in an animal breast tumor model.

Body

During the second year of funding, we moved along well with establishing our mammary gland tumor model with conditional activation of PIK3CA-H1047R. However, when we generated mice with mammary specific-loss of PTEN in BL6 background, female mice developed early onset of massive lymphomas, precluding further analysis of breast tumorigenesis. We found the possible cause and are taking alternative approaches. As both animal and cell culture work developed into a stage that required more devoted efforts, Dr. Wang, another post doctor fellow in the lab joined the project to carry out a new PTEN loss induced mammary tumor model and the final concurrent PTEN deletion and PIK3CA activation in mouse mammary gland. Details of the studies and results are as follows:

On Aim1: Inactivation of *PTEN* in mammary epithelial cells

As reported last year, we initially tried siRNA oligos against PTEN, but the transfection rate in human mammary epithelail cells (HMECs) was low (~10%). Using retro-viral based shRNA

(pSuper-retroviral-shPTEN) mediated knockdown, we generated HMEC lines stably transduced with three retroviral shPTENs targeting different regions of ***PTEN*** mRNA. Western-blot analysis showed that the best reduction of PTEN level was about 60% as compared to that of control cells. We found the 60% reduction of PTEN level was not sufficient to induce anchorage-independent growth of HMECs in the presence of hTERT, high level of c-my and p53DD. We then started working with lentiviral shRNAs against PTEN, pLKO-shPTEN, in HMECs and found two lenti-shPTENs were able to knockdown ~90% of endogenous PTEN (Figure. 1). Interestingly, whereas PIK3CA-H1047R induced robust colony formation of HMECs expressing hTERT, myc and p53DD, shPTEN could not substitute PIK3CA-H1047R for the same assay. It is possible that small amount of PTEN can protect cell from transformation and the ~10% residual PTEN in HMECs expressing shPTEN could be a reason for the failed colony formation in our assay.

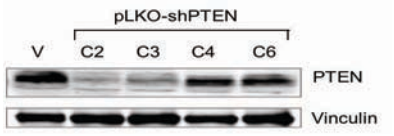

Figure 1. Endogenous PTEN knockdown in HMECs with pLKO-shPTENs.

We are now taking two alternative approaches: i) Use human breast cancer cell lines harboring PTEN loss, e.g. BT549, and introduce them with PIK3CA-H1047R or E545K construct. ii) Isolate floxed PTEN mouse mammary epithelial cells (MMECs) and introduce PIK3CA-H1047R or E545K construct into these cells before or after adeno-Cre mediated PTEN deletion event. These approaches will not only help us to resolve the potential problem of incomplete PTEN deletion from an shPTEN mediated gene knockdown, but also provide complementary systems to HMECs cell culture in vitro as well as animal mammary gland in vivo studies.

Regarding construction of an inducible shPTEN vector, we wanted to develop a single vector configuration for both regulatable shRNA mediated gene knockdown and expression of rtTA component to avoid cumbersome multi-steps of virus infection. In the middle of this undertaking, we found that a similar vector was generated by Dr. Melvin Simon's group (Shin et al., 2006) and is available from the American Type Culture Collection (ATCC). Since the TRE is a pol II promoter that can drive expressions of both mir-shRNA and ORF, we plan to use this system to generate an inducible shPTEN and an inducible PIK3CA-H1047R constructs as shown in Figure 2.

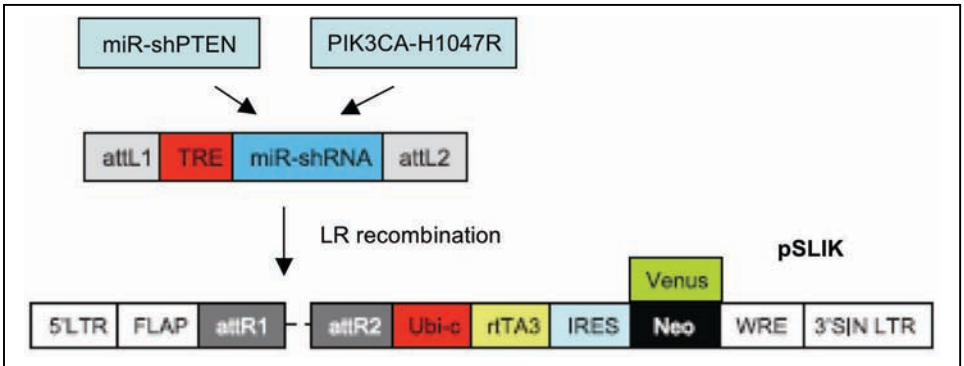

Figure 2. A diagram for making inducible shPTEN and PIK3CA-H1047R into pSLIK vector via gateway recombination cloning strategy.

On Aim 2: Simultaneous inactivation of PTEN and activation of PIK3CA in a mouse mammary tumor model

Activation of PIK3CA-H1047R induce mammary tumorigenesis: During the first year, we made an inducible transgenic construct "TetO-HA-PIK3CA-IRES-luciferase", which was then injected into embryos to produce a transgenic strain in FVB background carrying the inducible PIK3CA-H1047R-luc (T-H1047R-luc) transgene. Six positive founders were identified. Three founders (T-2222, T-2239, and T-2251) were bred with wild-type FVBs and produced progenies carrying the transgene. One founder (T-2241) was not able to pass the transgene to its progenies. Two founders (T-2216, T-2248) were discarded after being in breeders for four months without production. Last year, we crossed our T-H1047R-luc mouse with an MMTV-rtTA (MTB) line from Dr. Chodosh'lab (Gunther et al., 2002) and generated bi-transgenic mice, T-H1047R-luc/MTB. We found that the induced mammary glands of bitransgenic mice displayed hyperplasias following doxycycline induction for 6 days and mammary tumors for as early as 7 weeks (Figure 3 and data not shown). Our initial analysis found all three lines, T-2222, T-2239, and T-2251, having similar mammary neoplasia phenotype when they were crossed with MTB mice, and among them T-2239 bred well and showed robust signals. We are now focusing on this T-2239 line for our further characterization of the PIK3CA-H1047R activation in mammary tumor initiation, maintenance and progression, and the interaction with PTEN loss in mammary gland.

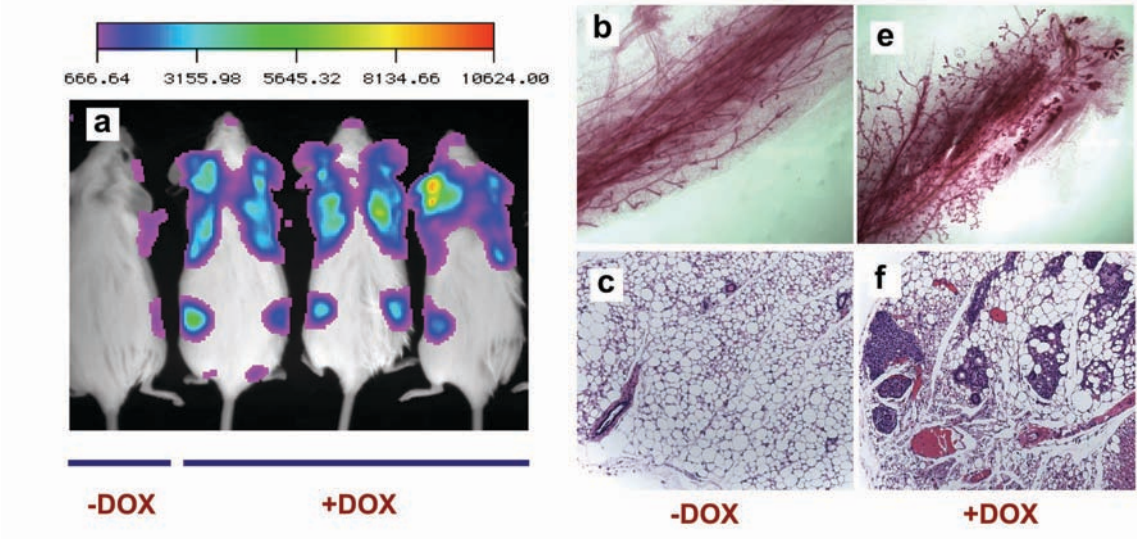

Figure 3. Doxycycline-dependent expression of H1047 and neoplasia in mammary gland. **a.** Luciferase imaging of bitrangenic T-H1047R-luc/MTB mice with or without doxycycline treatment. Whole-mount glands from mouse without doxycycline (**b**) and mouse on doxycycline (**e**). Hematoxylin and eosin (H&E) stained sections of mammary glands from mouse without doxycycline (**c**) and mouse on doxycycline (**f**). All mice used here are 7 weeks virgin T-H1047R-luc/MTB mice without or with doxycycline for 7 weeks.

Re-establishing mammary tumorigenesis induced by PTEN loss: Li *et al.* generated mammary gland-specific PTEN deletion mice (Li et al., 2002) by mating PTEN$^{flox/flox}$ mice with MMTV-Cre transgenic mice (Wagner et al., 1997). Female PTEN$^{flox/flox}$/MMTV-Cre mice developed tumors as early as 2 months and the mean latency was 9 to 10 months. We crossed the same BL6 MMTV-Cre line (Wagner et al., 1997) with a floxed PTEN mouse on BL6 background (Lesche et al., 2002). We found that all female PTEN$^{flox/flox}$;MMTV-Cre mice developed massive lymphomas between 2-3 months (Figure 4), precluding the analysis of the PTEN loss in mammary gland neoplasia. The discrepancy of the two studies is likely due to a difference on strain background. Both MMTV-rtTA and PTEN$^{flox/flox}$ lines were on BL6 background in our study, whereas *Li et al.* might have crossed BL6 MMTV-rtTA with Balb/c PTEN$^{flox/flox}$ in their reported work (Li et al., 2002) (Note: the background of the PTEN$^{flox/flox}$ line used in this particular study was not stated in the publication).

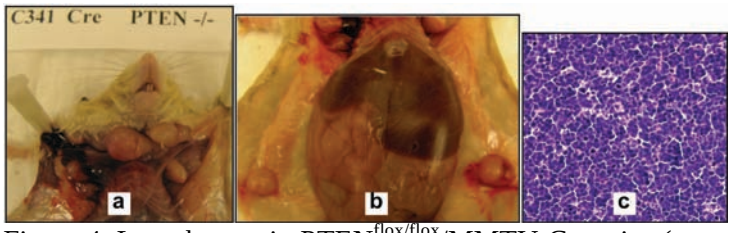

Figure 4. Lymphomas in PTEN$^{flox/flox}$/MMTV-Cre mice (**a** and **b**) and H&E stained section of a lymphoma (**c**) isolated from the 4th mammary gland (**b**).

In order to establish another PTEN loss induced mammary tumor model that is more suitable for our proposed study, we are backcrossing both MMTV-rtTA and PTEN$^{flox/flox}$ lines to FVB background and they are now at 4th generation. Since the incidences of lymphoma formation in heterozygous PTEN knockout mice depend on genetic background, e.g. ~90% on BL6 and ~10% on Balb/c background (Freeman et al., 2006), our current approach will allow us to determine whether FVB would be a good background to study PTEN loss in mammary gland function. As our T-H1047R/MMTV-rtTA mouse is on FVB background, backcrossing MMTV-rtTA and PTEN$^{flox/flox}$ lines to FVB will also facilitate our study on the same mouse genetic background.

It is known that expression of MMTV-Cre is not confined to the mammary gland, our female PTEN$^{flox/flox}$;MMTV-Cre mice on FVB background may still develop severe lymphoma or other types of tumor which will interfere our study on mammary tumorgenesis. In this case, we will use a transgenic mouse carrying Cre expression driven by WAP promoter. In fact the transgenic mouse carrying WAP-rtTA-Cre developed by Utomo *et al.* (Utomo et al., 1999) that integrates tetracycline-controlled gene expression and Cre-mediated gene deletion would be well suited for our study, especially for the final simultaneous activation of PIK3CA and inactivation PTEN. Luckily, we have this line in hand and it is at the 7th generation of backcrossing to FVB background. Importantly, using a single WAP-rtTA-Cre line to replace both MMTV-Cre and MMTV-rtTA lines to generate our final compound mice, namely T-H1047R-Luc/PTEN$^{lox/lox}$/WAP-rtTA-Cre, instead of T-H1047R-Luc/PTEN$^{lox/lox}$/MMTV-rtTA/MMTV-Cre, will significantly reduce our crossing time and efforts. In addition, the integrated expression of rtTA and Cre under a single WAP promoter will eliminate the concerns of the mosaic expression rtTA and Cre from two separate MMTV promoters, allowing us to better examine the simultaneous inactivation of PTEN and activation of PIK3CA in mammary gland tumorigenesis.

Key Research Accomplishments

1. Generated stable HMEC-shPTEN lines.
2. Generated a bi-transgenic mouse, T-H1047R-luc/MTB, in FVB background and found that conditional expression of PIK3CA-H1047R induced mammary gland tumor formation.
3. Generated a bi-transgenic line $PTEN^{flox/flox}$/MMTV-Cre in BL6 background, and found that female mice developed massive lymphomas within 2-3 months, suggesting that selecting an appropriate mouse strain background is important for a tissue-specific tumor genesis study.
4. Backcrossing floxed PTEN, MMTV-Cre and WAP-rtTA-Cre lines to FVB background, which will be useful strains for mammary gland biology and other in vivo animal studies.

Reportable Outcomes

The support from the Department of Defense Idea Award has helped me to start my independent research program and provided research opportunities for two postdoctoral fellows, Hailing Cheng and Theresa Wang, to pursue their careers in cancer research. Other outcomes, such as cell lines and animal models, are listed under "key research accomplishments"

Conclusion

The research described here is relevant to the pathogenesis and a potential novel therapy for breast cancers. The *PIK3CA* is the most commonly mutated oncogene in breast cancer and loss of the tumor suppressor, PTEN, occurs frequently in patients suffering from this disease. Our newly generated oncogenic *PIK3CA* transgenic animal model will allow us to determine the oncogenic role of *PIK3CA* in tumor initiation, progression, maintenance and metastasis etc. It should also significantly facilitate preclinical testing for the development of PI3K inhibitors for targeted therapy. Our final goal of simultaneous inactivation of *PTEN* and activation of *PIK3CA* will not only provide a new perspective on the relationship of the two key oncogene and tumor suppressor, *PIK3CA* and *PTEN*, and the signaling pathway under their control in cell regulation and oncogenic transformation, but also a potential novel therapy to all patients plagued with the common tumorigenic mutations.

References

Bachman, K. E., Argani, P., Samuels, Y., Silliman, N., Ptak, J., Szabo, S., Konishi, H., Karakas, B., Blair, B. G., Lin, C., *et al.* (2004). The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther *3*, 772-775.

Broderick, D. K., Di, C., Parrett, T. J., Samuels, Y. R., Cummins, J. M., McLendon, R. E., Fults, D. W., Velculescu, V. E., Bigner, D. D., and Yan, H. (2004). Mutations of PIK3CA in anaplastic

oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. Cancer Res *64*, 5048-5050.

Byun, D. S., Cho, K., Ryu, B. K., Lee, M. G., Park, J. I., Chae, K. S., Kim, H. J., and Chi, S. G. (2003). Frequent monoallelic deletion of PTEN and its reciprocal associatioin with PIK3CA amplification in gastric carcinoma. Int J Cancer *104*, 318-327.

Freeman, D., Lesche, R., Kertesz, N., Wang, S., Li, G., Gao, J., Groszer, M., Martinez-Diaz, H., Rozengurt, N., Thomas, G., *et al.* (2006). Genetic background controls tumor development in PTEN-deficient mice. Cancer Res *66*, 6492-6496.

Gunther, E. J., Belka, G. K., Wertheim, G. B., Wang, J., Hartman, J. L., Boxer, R. B., and Chodosh, L. A. (2002). A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. Faseb J *16*, 283-292.

Lesche, R., Groszer, M., Gao, J., Wang, Y., Messing, A., Sun, H., Liu, X., and Wu, H. (2002). Cre/loxP-mediated inactivation of the murine Pten tumor suppressor gene. Genesis *32*, 148-149.

Li, G., Robinson, G. W., Lesche, R., Martinez-Diaz, H., Jiang, Z., Rozengurt, N., Wagner, K. U., Wu, D. C., Lane, T. F., Liu, X., *et al.* (2002). Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. Development *129*, 4159-4170.

Saal, L. H., Holm, K., Maurer, M., Memeo, L., Su, T., Wang, X., Yu, J. S., Malmstrom, P. O., Mansukhani, M., Enoksson, J., *et al.* (2005). PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res *65*, 2554-2559.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J., *et al.* (2004). High frequency of mutations of the PIK3CA gene in human cancers. Science *304*, 554.

Shin, K. J., Wall, E. A., Zavzavadjian, J. R., Santat, L. A., Liu, J., Hwang, J. I., Rebres, R., Roach, T., Seaman, W., Simon, M. I., and Fraser, I. D. (2006). A single lentiviral vector platform for microRNA-based conditional RNA interference and coordinated transgene expression. Proc Natl Acad Sci U S A *103*, 13759-13764.

Utomo, A. R., Nikitin, A. Y., and Lee, W. H. (1999). Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. Nat Biotechnol *17*, 1091-1096.

Wagner, K. U., Wall, R. J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P. A., and Hennighausen, L. (1997). Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res *25*, 4323-4330.

Appendices None